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Genetic Basis of Resistance to Rimantadine Emerging during Treatment of Influenza Virus Infection

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The emergence of influenza A viruses which had acquired resistance to rimantadine during a clinical trial (C. B. Hall, R. Dolin, C. L. Gala, D. M. Markovitz, Y. Q. Zhang, P. H. Madore, F. A. Disney, W. B. Talpey, J. L. Green, A. B. Francis, and M. E. Pichichero, Pediatrics 80:275–282, 1987) provided the opportunity to determine the genetic basis of this phenomenon. Analysis of reassortant viruses generated with a resistant clinical isolate (H3N2) and the susceptible influenza A/Singapore/57 (H2N2) virus indicated that RNA segment 7 coding for matrix and M2 proteins conferred the resistant phenotype. Resistant viruses isolated from seven patients each contained a single change in the nucleotide sequence coding for the M2 protein which resulted in substitutions in amino acid 30 (two viruses) or 31 (five viruses) in the transmembrane domain of the molecule. These changes occurred in locations identified in influenza viruses selected for resistance to amantadine in tissue culture and indicate a common mechanism of action of the two compounds in cell culture and during chemotherapeutic use.

Among the first clinically useful antiviral compounds are the adamantane derivatives, amantadine (1-aminoadamantane hydrochloride) and rimantadine (α-methyl-1-adamantane methylamine hydrochloride). These were recognized as having activity against influenza A viruses as early as 1964 (3), and amantadine was approved in 1966 by the U.S. Food and Drug Administration for use in the United States as an oral agent for the prevention and treatment of influenza A virus infections. Recently there has been renewed interest in rimantadine, which is as efficacious as amantadine but lacks the frequent, mild adverse reactions of light-headedness and inability to concentrate (4, 19, 22, 24, 25).

Amantadine-resistant influenza A viruses have been readily selected in animals (17) and in tissue culture (1) by growing the virus in the presence of the drug, and complete cross-resistance between amantadine and rimantadine has been observed. Although the limited clinical use of amantadine for the past 2 decades has not resulted in drug-resistant epidemic strains of influenza A virus, the availability of a second adamantane derivative for the treatment of influenza will place additional selective pressure on contemporary strains. It is important, therefore, to understand the genetic basis for resistance to amantadine and rimantadine.

The first well-documented isolates of drug-resistant influenza A virus (H3N2 and H1N1) emerging during treatment with rimantadine have recently been described (5, 21). Although previous reports of amantadine-resistant viruses isolated from patients have been made, these viruses have not been completely characterized (11, 18). The viruses characterized in this report were isolated during an efficacy study which compared oral rimantadine with acetaminophen in the treatment of children with documented influenza A H3N2 virus infection (5). Rimantadine-treated patients showed a significantly greater improvement within 2 days compared with acetaminophen-treated children. Among all children who were treated with rimantadine, 27% shed resistant virus during therapy, and among those shedding virus for 7 days, 45% shed resistant virus. The genetic basis of resistance to rimantadine was examined in isolates from seven patients, each of whom initially shed rimantadine-sensitive and subsequently shed rimantadine-resistant virus during therapy.

MATERIALS AND METHODS

Viruses. A total of 14 influenza A viruses (influenza A/H3N2/NY/83) from seven patients were selected for study. These seven pairs were chosen because in each instance the virus shed by an infected child prior to treatment was susceptible to rimantadine but the isolate obtained on day 4, 5, or 6 of therapy exhibited resistance to the action of rimantadine (5). Viruses had been isolated in cynomolgus monkey kidney or MDCK tissue culture cells and were in the second or third passage when passaged to fertilized hen eggs. Viral RNA for nucleotide sequence determination was extracted from virus obtained from allantoic fluid as previously described (2).

The parent susceptible isolate from each pair was passaged twice in MDCK cells in the presence of 1 μg of rimantadine per ml in order to select resistant viruses as previously described (7). The nucleotide sequence of RNA segment 7 from the resulting resistant viruses was determined in order to compare the in vivo resistant viruses with those generated in tissue culture.

Susceptibility tests. Susceptibility to rimantadine (1 μg/ml) was assayed by plaque titration as previously described (9). Results were confirmed by using an enzyme-linked immunosorbent assay (ELISA) of infected cells.

Rimantadine susceptibility of isolates was determined by ELISA as follows. MDCK cells in microdilution plates (quadruplicate wells) were infected with various dilutions of virus (allantoic fluid from infected 10-day-old fertile eggs) in Eagle medium plus 2.5 μg of trypsin per ml in the presence or absence of 1 μg of rimantadine per ml and incubated at 37°C for 16 h. Cells were fixed with 0.05% glutaraldehyde in phosphate-buffered saline (PBS) at room temperature for 15 min, washed, and incubated for 1 h at 37°C with 50 μl of a 10^3

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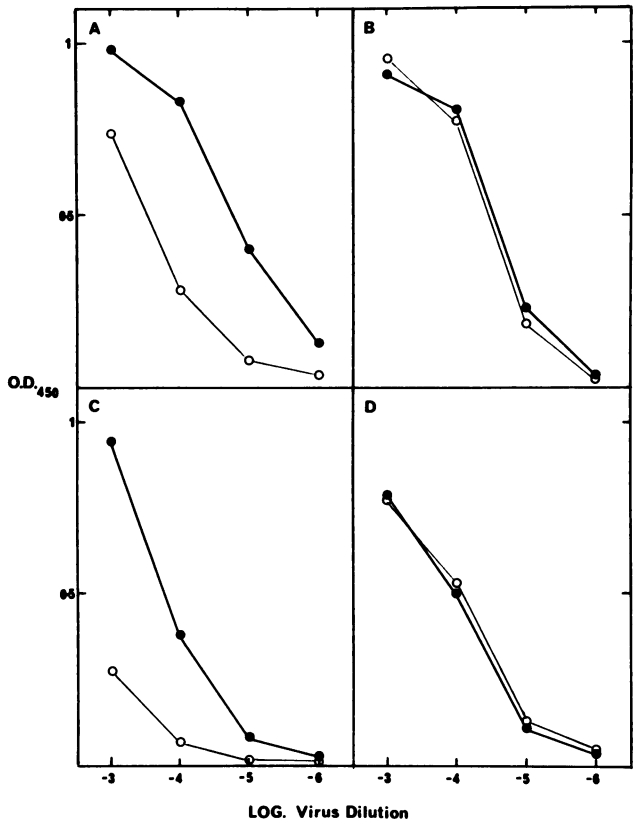

FIG. 1. Inhibition of the appearance of hemagglutinin on infected cell surfaces by rimantadine. Viruses in panels A and B were isolated from patient 19 on days 0 and 5, respectively, of rimantadine treatment, and viruses in panels C and D were isolated from patient 56 on days 0 and 4, respectively. Symbols: ●, control; ○, plus rimantadine.

dilution in PBS–0.5% bovine serum albumin of ferret antiserum raised against A/Mississippi/1/85. The cells were then washed and incubated for 1 h at 37°C with 50 μl of a 10^4 dilution of protein A-horseradish peroxidase conjugate (Bio-Rad Laboratories) in PBS–0.5% bovine serum albumin, washed with PBS, and incubated at room temperature for 2 to 5 min with 50 μl of 25 mM sodium citrate (pH 4.5) containing 0.02% 3,3',5,5'-tetramethylbenzidine dihydrochloride (Sigma Chemical Co.) and 0.01% hydrogen peroxide. The reaction was stopped by the addition of 50 μl of 1 M H_2SO_4, and optical densitities at 450 nm were determined.

Reassortants and nucleotide sequence determination. Reassortant viruses were generated by coinfecting primary chick kidney tissue culture cells with a plaque-purified resistant isolate obtained from patient 19 (designated influenza A/NY/83/R6) and a susceptible virus, influenza A/Singapore/57 (H2N2), as previously described (7). The genome compositions of reassortant viruses were determined by hybridization analysis as reported previously (6).

Nucleotide sequences of virus M genes were determined by the dideoxynucleotide chain-terminating procedure of Sanger et al. (20) as described previously (7).

RESULTS

The determination of susceptibility or resistance to 1 μg of rimantadine per ml previously reported by Hall et al. (5) was confirmed by both plaque reduction and ELISA. The most definitive susceptibility data were obtained by using an ELISA to measure the appearance of hemagglutinin on infected cell surfaces, and results obtained for pairs of sensitive and resistant isolates from two patients are shown in Fig. 1. The multiplicity of infection was varied to span the most sensitive assay conditions, viz., during the productive phase of virus growth. This was preferable to varying the time of incubation from the point of view of simplicity of assay and to take account of differences in virus growth rate and infectivity of virus inocula. Viruses isolated on day 0 or day 1 of rimantadine treatment exhibited greater than five-fold reductions in virus growth under optimal conditions, whereas the growth of resistant viruses isolated on days 4 to 6 was not significantly affected by concentrations of rimantadine up to 10 μg/ml.

The virus genes responsible for conferring drug resistance were investigated by comparisons of the rimantadine susceptibilities and genome compositions of genetic reassortants produced following coinfection of cells with the resistant isolate from patient 19, designated influenza A/NY/83/R6 (H3N2), and a susceptible virus, influenza A/Singapore/57 (H2N2), as previously described (7) (Table 1). As in similar investigations with an amantadine-resistant virus isolated from tissue culture, gene 7 alone was the principal determinant of drug susceptibility (6). Thus, reassortant viruses containing RNA segment 7 from A/Singapore were sensitive to rimantadine regardless of the parental origin of the remaining genes, and, conversely, all reassortant viruses containing segment 7 from R6 were resistant. Since all resistant viruses examined also contained gene 2 of influenza A/NY/83/R6, it was not possible to rule out some influence of this gene, although, on its own, this gene had no discernible effect on susceptibility. It was apparent, therefore, that mutation of the M gene, which encodes the matrix (M1) protein and the smaller M2 protein translated from a spliced mRNA (13), was responsible for the loss of rimantadine sensitivity of R6.

The virus isolates obtained prior to rimantadine therapy fell into two groups—those from patients 7, 53, 86, and 90 and those from patients 19, 56, and 88—on the basis of differences in their M gene sequences, although they were not distinguishable antigenically in hemagglutination inhibition tests with ferret antisera prepared against a series of

TABLE 1. Rimantadine susceptibility of genetic reassortants of rimantadine-resistant virus A/NY/83/R6 (H3N2) and rimantadine-sensitive A/Singapore/1/57 (H2N2)

Rimantadine susceptibility and reassortant no.	Genome composition[a]							
	1	2	3	4	5	6	7	8
Sensitive								
1	S	S	S	N	N	N	S	N
8	S	N	S	N	N	N	S	N
10	S	N	N	S	S	S	S	S
12	N	N	N	S	S	N	S	S
17	S	N	N	N	S	S	S	N
18	N	N	S	N	S	S	S	N
Resistant								
2	S	N	S	S	S	N	N	N
3	S	N	N	S	S	N	N	N
4	N	N	S	S	S	N	N	S
6	N	N	S	N	S	S	N	N
11	N	N	S	S	S	N	N	N

[a] N and S, RNA segments derived from A/NY/83/R6 and A/Singapore/1/57 parent viruses, respectively.

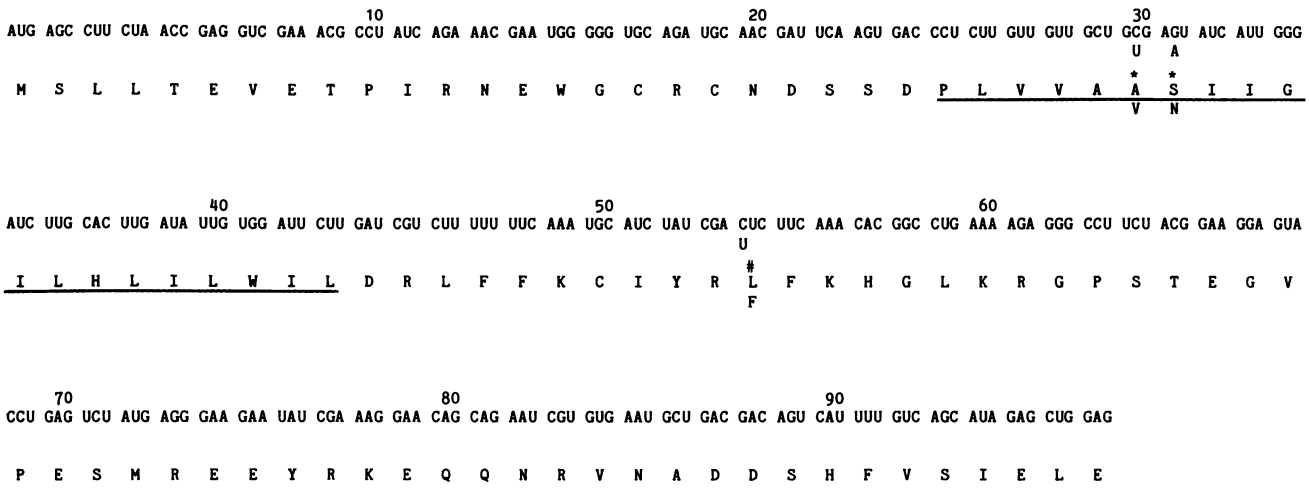

FIG. 2. Coding and deduced amino acid sequences of the M2 protein of rimantadine-sensitive and -resistant influenza A/NY/83 virus isolates. The spliced coding sequence of M2 is as predicted from the data of Lamb et al. (13). Symbols: *, mutations and amino acid substitutions in rimantadine-resistant viruses; #, leucine/phenylalanine difference at residue 54 between the two groups of rimantadine-sensitive viruses. The hydrophobic sequence of amino acids 25 to 43 is underlined.

H3N2 viruses isolated between 1977 and 1985. Of the seven nucleotide differences, two resulted in variance in amino acid sequence, one in the M1 protein and the other in the M2 protein at residue 54 (Fig. 2). To put the extent of the difference in perspective, 30 differences in nucleotide sequence accumulated over 11 years between the M genes of A/Udorn/72 (13) and A/NY/83, resulting in six amino acid changes, two in M1 and four in the M2 protein, reflecting the greater degree of variability observed in the later (12, 16).

The mutations encoding resistance were located by comparing the nucleotide sequences of RNA segment 7 of the resistant viruses isolated from patients after 4 to 6 days of rimantadine therapy with those of the corresponding sensitive viruses isolated on day 0 or day 1. Comparisons of the sequences of the M genes of corresponding sensitive and resistant isolates showed only a single nucleotide difference in each case, all of which result in an amino acid substitution in the M2 protein (Table 2 and Fig. 2). One isolate, from patient 19, was a mixture of viruses with two phenotypes, only 50% of which were resistant to rimantadine in the plaque assay; plaque-purified resistant virus was shown unambiguously to contain the codon for Asn-31. It is evident, therefore, that replacement of Ala-30 by valine in M2 of two viruses and Ser-31 by asparagine in M2 of the other five is responsible for the resistant phenotype.

Rimantadine-resistant variants were also isolated from six of the initial sensitive viruses by passage through MDCK cells in the presence of 1 μg of rimantadine per ml. Four of these, from patients 5, 7, 9, 86, and 88, contained the same alteration in the M2-coding sequence as did the corresponding isolates from resistant viruses selected in vivo. The variant selected from virus 5, obtained from patient 19, also differed from the corresponding resistant virus 6 in having Val-27 substituted by alanine, and the variant selected from the day 0 isolate from patient 53 contained asparagine at residue 31. It is evident, therefore, that the rimantadine-resistant mutants selected in cell culture are similar to those emerging in children receiving rimantadine therapy.

DISCUSSION

These observations extend those made on amantadine-resistant avian and human influenza A viruses selected in tissue culture by passage in the presence of amantadine, which indicated that the M2 protein is the major deteminant of susceptibility to the action of amantadine (7, 8). The sequences of all amantadine-resistant viruses determined to date have contained single changes in the amino acid sequence of the transmembrane portion of M2 (7, 14). These have occurred at residue 27 (40 viruses), 30 (25 viruses), 31 (18 viruses), or 34 (29 viruses). The genetic change to amino acid 31 in the majority of the isolates from rimantadine-treated patients and those selected from cell culture was the same as that seen with half of the amantadine-resistant influenza A/Singapore/57 (H2N2) viruses (7). More recently, direct comparisons of drug-resistant variants of A/Singapore selected by passage in MDCK cells in the presence of 1 μg of amantadine or rimantadine per ml have indicated a similar proportion of viruses with the same amino acid changes in residues 27 (Val→Ala) and 31 (Ser→Asn). One difference from previous observations is in the substitution of Ala-30 by

TABLE 2. Correlation of amino acid substitutions in the M2 protein with sensitivity or resistance of clinical isolates to rimantadine

Patient no.	Day of treatment[a]	Susceptibility of isolate[b]	Amino acid substitution in M2/residue no.
7	0	S	Ser→Asn/31
	5	R	
19	0	S	Ser→Asn/31
	5	R	
53	0	S	Ala→Val/30
	5	R	
56	0	S	Ala→Val/30
	4	R	
86	0	S	Ser→Asn/31
	4	R	
88	1	S	Ser→Asn/31
	4	R	
90	1	S	Ser→Asn/31
	6	R	

[a] Day of treatment indicates the day of rimantadine therapy on which respiratory secretions were obtained for isolation of influenza A virus. Zero indicates pretreatment isolate.

[b] S, Sensitive; R, resistant.

valine in patients 53 and 56; in amantadine-resistant variants selected in cell culture substitution was by threonine or, less frequently, by proline (7).

From these data it is evident, therefore, that the genetic basis of resistance to rimantadine in viruses emerging during clinical use of the compound is the same as that in viruses selected in tissue culture and that the actions observed in cells (7, 8) reflect the activity in humans. These observations confirm that the mechanism of action of rimantadine is the same as that of amantadine and explain the observed cross-resistance that occurs in tissue culture. Furthermore, these findings also indicate that cross-resistance will occur during chemotherapeutic use of the drugs.

The action of amantadine on viral replication varies according to virus strain; most influenza A viruses are inhibited early during the initiation of infection, whereas some avian influenza viruses are inhibited at a later stage, preventing virus assembly. The data discussed above have pointed to the M2 protein as the primary target of rimantadine action; however, the hemagglutinin has also been implicated in the action of amantadine (14). Amantadine treatment of cells infected with certain avian viruses causes an alteration in the maturation of the hemagglutinin during transport to the cell surface (A. J. Hay and R. Sugrue, manuscript in preparation). The hemagglutinin expressed on the surfaces of these cells has a conformation similar to that following low pH treatment (2), and as a consequence, virus production is prevented. Since changes in the amino acid sequence of M2 alone are capable of abolishing this effect of the drug, its action against the hemagglutinin is apparently indirect and may result from interference with interactions occurring between the M2 and hemagglutinin proteins. For most viruses examined, including human isolates, however, the principal block to replication occurs at a stage during virus entry into cells. In view of the similarities in the amino acid substitutions in M2 which confer resistance to both early and late actions of the drug and by analogy with the phenomenon described above, the early inhibition of virus replication may also result from the indirect impairment of hemagglutinin function, e.g., membrane fusion involved in virus uncoating. In this regard the rate of membrane fusion in vitro by these viruses is specifically reduced by similar low drug concentrations of approximately 5 μg/ml (S. Wharton, R. B. Belshe, and A. J. Hay, manuscript in preparation).

The isolation of drug-resistant viruses from a high proportion of the children treated with rimantadine correlates with the relative ease with which resistant viruses are isolated from tissue-culture-grown virus populations, with a frequency of 10^{-3} to 10^{-4} (1, 15). Children, however, tend to shed virus longer and in larger quantities than adults do (10) and therefore may be more likely to shed drug-resistant virus during treatment. Furthermore, the majority of viruses shed late in the course of therapy had resistant phenotypes as indicated by plaque assay and nucleotide sequence determination; only one isolate obviously contained a mixture of sensitive and resistant viruses.

What, therefore, are the implications for the development of drug-resistant epidemic strains in the human population? Since the amino acid changes in M2 completely abolish susceptibility, extensive use of rimantadine might be expected to lead to the spread of resistant virus, as has occurred under field conditions in chickens (23). However, we have at present little information on the biological characteristics of rimantadine-resistant viruses which might influence their viability. Although resistant variants in cell culture replicate equally well in the presence or absence of drug, this may not be so in vivo, and they may have a reduced disease potential, as do, for example, certain acyclovir-resistant herpesviruses, which may limit their ability to compete effectively with cocirculating sensitive strains. It may be more than coincidence that most natural isolates examined are sensitive to these drugs. Furthermore, there is no evidence to indicate that the limited use of amantadine and rimantadine to date has given rise to significant resistant viruses in the human population. Although it is apparent that in cell culture changes in the virus hemagglutinin as a result of genetic reassortment can substantially reduce susceptibility to amantadine (8), all subtypes of human influenza A viruses are sensitive to these drugs at pharmacologic concentrations. Finally, a feature which may mitigate the problem of acquired drug resistance in influenza viruses is the continual selection of antigenic variants. Thus, if prescribing of amantadine and rimantadine is limited only to those persons with a medical indication for prevention of influenza A or to treatment of persons with influenzalike illness during documented periods of virus activity in the community, the chance of an antigenically distinct epidemic strain emerging which also contains a rimantadine-resistant M2 protein should be minimized.

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